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The *Hansenula polymorpha per6* mutant is affected in two adjacent genes which encode dihydroxyacetone kinase and a novel protein, Pak1p, involved in peroxisome integrity

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Abstract The *Hansenula polymorpha per6-210* mutant is impaired in respect of growth on methanol (Mut⁻) and is characterized by aberrant peroxisome formation. The functionally complementing DNA fragment contains two open reading frames. The first encodes dihydroxyacetone kinase (*DAK*), a cytosolic enzyme essential for formaldehyde assimilation; the second ORF codes for a novel protein (Pak1p). We have demonstrated that *per6-210* cells lack DAK activity, causing the Mut⁻ phenotype, and have strongly reduced levels of Pak1p, resulting in peroxisomal defects. Sequence analysis revealed that *per6-210* contains a mutation in the 3′ end of the *DAK* coding region, which overlaps with the promoter region of *PAK1*. Possibly this mutation also negatively affects *PAK1* expression.

Key words Peroxisome biogenesis · Methanol metabolism · Yeast

Introduction

Peroxisomes are essential for growth of methylotrophic yeasts on methanol as the sole source of carbon and energy. These organelles harbour the key enzymes of methanol metabolism: alcohol oxidase (AO), dihydroxyacetone synthase (DHAS) and catalase (CAT). Other enzymes of the dissimilatory (formaldehyde and formate dehydrogenase) and assimilatory (the xylulose-5-phosphate) pathways are located in the cytosol (reviewed by Veenhuis and Harder 1988).

In order to identify the genes essential for the growth of methylotrophic yeasts on methanol, various methanol-utilization-deficient (Mut⁻) mutants of the yeast *Hansenula polymorpha* have been isolated (De Koning et al. 1987; Cregg et al. 1990). Within these collections different classes of Mut⁻ strains have been discriminated, namely: (1) strains mutated in structural genes encoding peroxisomal or cytosolic enzymes involved in methanol metabolism (Verduyn et al. 1984; de Koning et al. 1987), and (2) *pex* mutants, which are defective in peroxisome biogenesis and/or function (Cregg et al. 1990). In these mutants the Mut⁻ phenotype is due to the mislocation of enzymatically active peroxisomal enzymes to the cytosol (van der Klei et al. 1991).

Here, we describe the *H. polymorpha per6-210* mutant, which has been identified within a previously described collection of *H. polymorpha* Mut⁻ mutants (Titorenko et al. 1993). We found that this mutant is deficient in an enzyme required for methanol metabolism, but is also disturbed in peroxisome formation. We show that a single mutation in *per6-210* affects the protein products of two adjacent genes. The first encodes the enzyme dihydroxyacetone kinase (DAK), a cytosolic enzyme essential for the assimilation of formaldehyde produced from methanol oxidation. The second ORF, designated *PAK1*, encodes a novel protein. The peroxisomal defect in *per6-210* is most probably due to strongly reduced levels of Pak1p.

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Materials and methods

Organisms and growth conditions. H. polymorpha wild-type (CBS 4732), the *per6-210* mutant (Titorenko et al. 1993), NCYC 495 *leu1.1* (Waterham et al. 1994), NCYC 495 *leu1.1 ura3* (Waterham et al. 1994), and the constructed disruption strains (see below) were grown

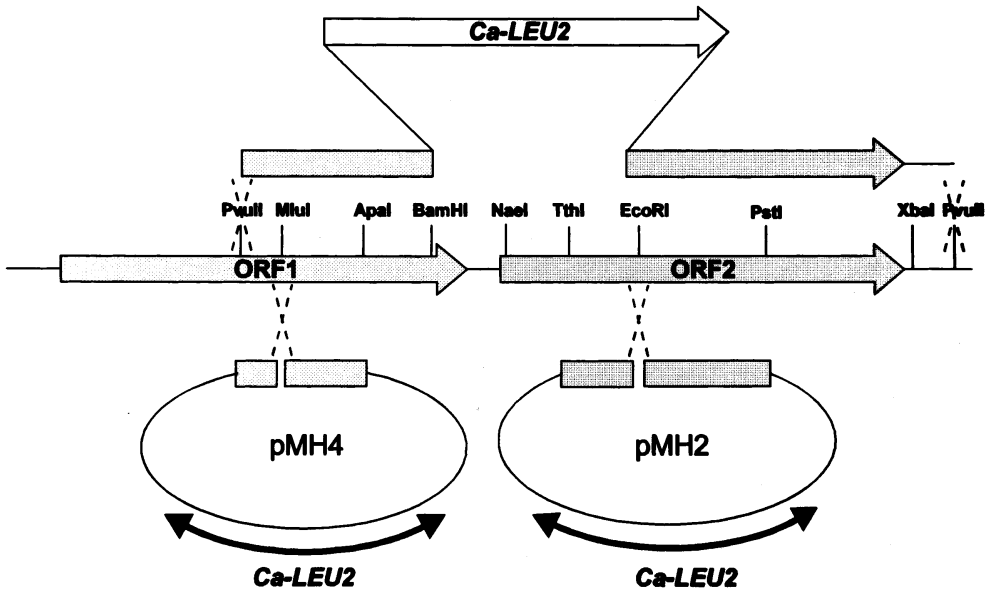

Fig. 1 Schematic representation of the *per6-210* complementing DNA fragment. The *dotted arrows* represent ORF1 and ORF2. Construction of the Δ*dak* and Δ*pak1* strains by integration of plasmids pMH4 in ORF1 or pMH2 in ORF2 are also indicated. The Δ*dak* Δ*pak1* double mutant was constructed by replacing parts of ORF1 and ORF2 by a DNA fragment containing *C. albicans LEU2* (see Materials and methods section). Only relevant restriction sites are indicated

at 37°C on rich complex medium (YPD) containing 1% yeast extract, 2% peptone and 1% glucose, on mineral media as described (van Dijken et al. 1976), or on YNB without amino acids containing 0.67% Yeast Nitrogen Base (Difco). The carbon sources used were 0.5% glucose, 0.5% ethanol, 0.5% glycerol, 0.5% dihydroxyacetone or 0.5% methanol; as nitrogen sources ammonium sulphate or methylamine (both at 0.2%) were employed. Carbon-limited continuous culturing was carried out as described previously (van Dijken et al. 1976) at a dilution rate of 0.05 h^{-1}. Amino acids and uracil were added to a final concentration of 30 μg/ml. To mineral media the vitamins thiamine (20 μg l^{-1}) and biotin (600 μg l^{-1}) were added, unless indicated otherwise. *Escherichia coli* strains DH5α (GIBCO-BRL, Gaithersburg, Md.) and C600 (Stratagene, La Jolla, Calif.) were grown at 37°C in LB medium or in minimal M9 medium (Sambrook et al. 1989), supplemented with ampicillin (100 μg/ml) or kanamycin (50 μg/ml) when required.

Miscellaneous DNA techniques. Standard recombinant DNA techniques, *E. coli* transformation and plasmid isolation were performed essentially as described (Sambrook et al. 1989). *H. polymorpha* was transformed by electroporation (Faber et al. 1994).

Cloning and characterization of the complementing genes. H. polymorpha per6-210 was transformed with a genomic DNA library of *H. polymorpha* (Waterham et al. 1994). A plasmid containing a complementing fragment of approximately 5.2 kb was isolated. DNA fragments comprising 4.4 kb of the insert were subcloned in pBluescript (Stratagene, La Jolla, Calif., USA) and sequenced. Nested deletions were generated using Exonuclease III and S1 nuclease as described (Sambrook et al. 1989). Double-stranded sequencing was performed by the dideoxy chain-termination method (Sanger et al. 1977). For analysis of the DNA and amino-acid sequences the PC-GENE™-program, release 6.70 (IntelliGenetics Inc.), was employed. The BLASTN, BLASTP and TBLASTN algorithms (Altschul et al. 1990) were used to screen the GenBank database and the *Saccharomyces* Genome Database (SGD, Stanford University, Calif.) for proteins having similarity to the predicted gene products. In order to identify the mutation in *per6-210* a DNA fragment, including the 3′ end of ORF1, where the mutation was expected to be located (see Results section), was sequenced. For this purpose a PCR reaction was performed using a proof-reading enzyme and chromosomal DNA isolated from the *per6-210* mutant. The following primers were used: 5′-CTC GTC GGA CGA GGT TGT GC-3′ (nucleotides 1269–1288) and 5′-CGG TGA TGG TCT TCA CGT CC-3′ (nucleotides 2480–2499 in GenBank accession no. AF061946). From the resulting PCR product a 1.1-kb *XhoI-NaeI* fragment (nucleotides 1321–2442, comprising the 3′ end of *DAK* and the 5′ end of *PAK1*) was cloned into pBluescript II SK^+ (Stratagene) and sequenced.

Gene disruptions. For the disruption of the *H. polymorpha DAK* and *PAK1* genes we constructed the integration plasmids pMH2 and pMH4, containing internal fragments of either one of the genes. Plasmid pMH2 was constructed as follows. First a 770-bp *Tth*I(blunted)-*Pst*I fragment of *PAK1* (nucleotides 2669–3441 in GenBank accession no. AF061946) was cloned into *Hinc*II+*Pst*I-digested pBluescript II SK^+, resulting in plasmid pMH1. Subsequently a 2.2-kb (*Eco*RI-*Bam*HI)(blunted) fragment containing the *Candida albicans LEU2* gene (obtained from Dr. E. Berardi, University of Ancona, Ancona, Italy) was inserted in the *Sma*I site of pMH1. Plasmid pMH4 was constructed in a similar way. First a 650-bp *Pvu*II-*Apa*I internal fragment from the *DAK* gene (nt 1357–2003) was ligated between the *Eco*RV and *Apa*I sites of pBluescript II SK^+ resulting in plasmid pMH3. Subsequently, a 2.2-kb *Eco*RI-*Bam*HI fragment, carrying the *C. albicans LEU2* gene, was ligated between the *Eco*RI and *Bam*HI sites of pMH3. Plasmids pMH2 and pMH4 were digested with *Eco*RI and *Mlu*I in the *PAK1* and *DAK* regions, respectively, and integrated in the *H. polymorpha NCYC 495 leu1.1 ura3* genome (Faber et al. 1994). A Δ*dak* Δ*pak1* double mutant, in which both the 3′ end of *DAK* (coding for amino acids 587–609) and the 5′ end of *PAK1* (coding for amino acids 1–167) were deleted, was constructed by directed integration using a *DAK-PAK1 Pvu*II fragment (nt 1357–4422) in which the *Bam*HI-*Eco*RI portion (nt 2208–2828) was replaced by a 2.2-kb *Eco*RI-*Bam*HI fragment containing the *C. albicans LEU2* gene (Fig. 1). In all cases leucine prototrophic transformants were checked for the proper insertion into the *H. polymorpha* genome by Southern analysis, using the ECL gene detection system (Amersham International, Amersham, UK) as recommended by the supplier (data not shown).

Construction of a Pak1p over-producing strain. Overexpression of *PAK1* was achieved as follows. First, a *Bam*HI site was introduced upstream of the first start codon of *PAK1* by PCR (5′ primer: TGT GGA TCC AAA ATG GAG GCC TTT G). A 2.1-kb *Bam*HI-XhoI fragment containing *PAK1* was cloned into *Bam*HI-*Sal*I-digested pHIPX4-B (Komori et al. 1997). The resulting plasmid was linearized with *Sph*I and integrated into the genome of *H. polymorpha* NCYC495 *leu1.1*. Correct integration in the P_{AOX} locus as a single copy was confirmed by Southern-blot analysis (data not shown).

Biochemical methods. Crude extracts were prepared as described (Waterham et al. 1994). For the generation of protoplasts whole cells were pre-incubated in a solution containing 100 mM Tris pH 8.0,

50 mM EDTA, 140 mM β-mercaptoethanol and 1.2 M sorbitol for 15 min at 37°C. The cells were harvested by centrifugation, washed once in a 50 mM potassium phosphate buffer pH 7.2, containing 1.2 M sorbitol, and incubated in the same buffer containing 1 mg ml^{-1} of zymolyase 20 T at 37°C for 30–120 min. All subsequent steps were performed at 4°C. Protoplasts were collected by centrifugation, washed in 5 mM MES pH 5.5 containing 1.2 M sorbitol, 1 mM PMSF, 5 mM NaF and 2.5 μg ml^{-1} of leupeptin, and subsequently homogenized using a Potter Elvehjem homogenizer. The homogenate was centrifuged for 10 min at 3000 g. The resulting supernatant was subsequently centrifuged for 10 min at 4000 g. The resulting post-nuclear supernatant (PNS) was either centrifuged for 30 min at 30 000 g, in order to obtain an organellar pellet, or directly loaded onto a discontinuous sucrose density gradient (Douma et al. 1985). High-salt treatment of the organellar pellet was carried out as described by Komori et al. (1997). Protein concentrations were determined according to Bradford (1976) using bovine serum albumin as a standard. Dihydroxyacetone kinase was assayed as described by Bystrykh et al. (1990). DHA (Bystrykh et al. 1990) and methanol concentrations (Verduyn et al. 1984) were assayed by established procedures. SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). Gels were used for Western blotting (Kyhse-Anderson 1984), and blots were decorated using the chromogenic or chemiluminescent Western blotting kit (Boehringer Mannheim, Germany) with specific polyclonal antibodies against *H. polymorpha* proteins.

Generation of polyclonal Pak1p antibodies. A 1.8-kb *Nae*I-*Xba*I fragment of *PAK1* (encoding amino acids 40–592) was cloned between the *Xmn*I and *Xba*I sites of pMAL-C2 (New England Biolabs), resulting in a fusion gene consisting of the *E. coli malE* gene and *PAK1*. Expression of the fusion gene in *E. coli* was induced by adding 1 mM of IPTG to exponentially growing cultures. After 2 h of incubation the cells were harvested and lysed using lysozyme. The fusion protein was recovered by centrifugation as inclusion bodies, which were used for immunization in rabbit (Harlow and Lane 1988).

Electron microscopy. Whole cells and spheroplasts were fixed and embedded in Epon 812 or Unicryl as described previously (Waterham et al. 1994). Ultrathin Unicryl-sections were labelled using polyclonal antibodies raised in rabbit and goat-anti-rabbit antibodies conjugated to gold according to the instructions of the manufacturer (Amersham, UK).

^{31}P nuclear magnetic resonance (NMR) experiments. ^{31}P NMR spectra were obtained at 121.49 MHz on a Bruker MSL 300 spectrometer according to the procedures detailed previously (Nicolay et al. 1987).

Results

Characterization of the *per6-210* mutant

The *H. polymorpha per6-210* mutant has been identified within a collection of methanol-utilization-deficient (Mut^-) strains detailed previously (Cregg et al. 1990; Titorenko et al. 1993). Cells of this strain normally grow on rich media (e.g. YPD, mineral media containing glucose, ethanol or glycerol). However, growth on methanol is fully impaired. Upon transfer of glucose-grown *per6-210* cells to methanol-containing media, peroxisomes developed which displayed an aberrant morphology (data not shown). Electron microscopical analyses showed that, after a shift of cells from glucose to methanol-containing media and due to the import of induced peroxisomal proteins [e.g. alcohol oxidase (AO), dihydroxyacetone synthase (DHAS) and catalase], the initial development of the organelles was virtually unaffected and occurred as described for WT cells. However, upon maturation of the organelles (during prolonged incubation) their surrounding membranes frequently became ruptured. This process was associated with the formation of several new small organelles, which were virtually intact and, since they grew, were able to import newly synthesized proteins. The remaining, damaged organelles were often subject to rapid autophagic degrada-

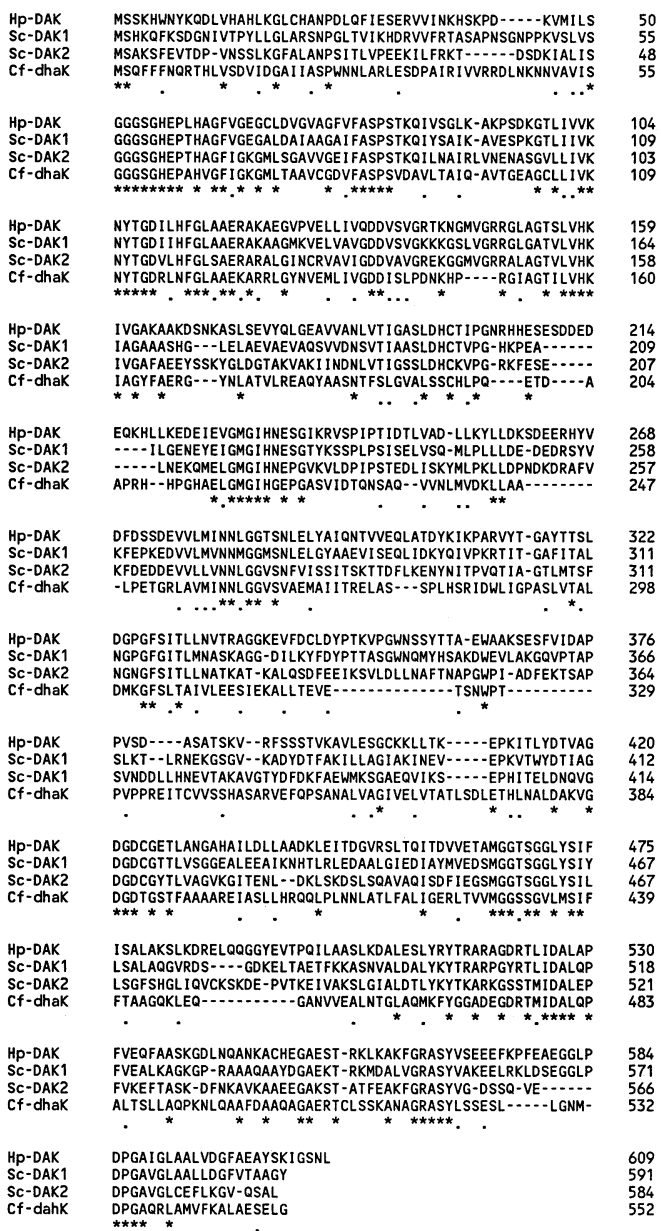

Fig. 2 Comparison of the deduced amino-acid sequences of *H. polymorpha DAK*, two homologous genes from *S. cerevisiae*, designated *DAK1* (Swiss-Prot P43550) and *DAK2* (PIR accession number S48327), and the *S. typhimurium dhaK* gene (Daniel et al. 1995). The proteins are homologous over their entire length. Identical amino acids are indicated by an *asterisk*, similar ones by a *dot*. In *per6-210* the conserved glycine at position 401 has changed into glutamic acid (401G-E)

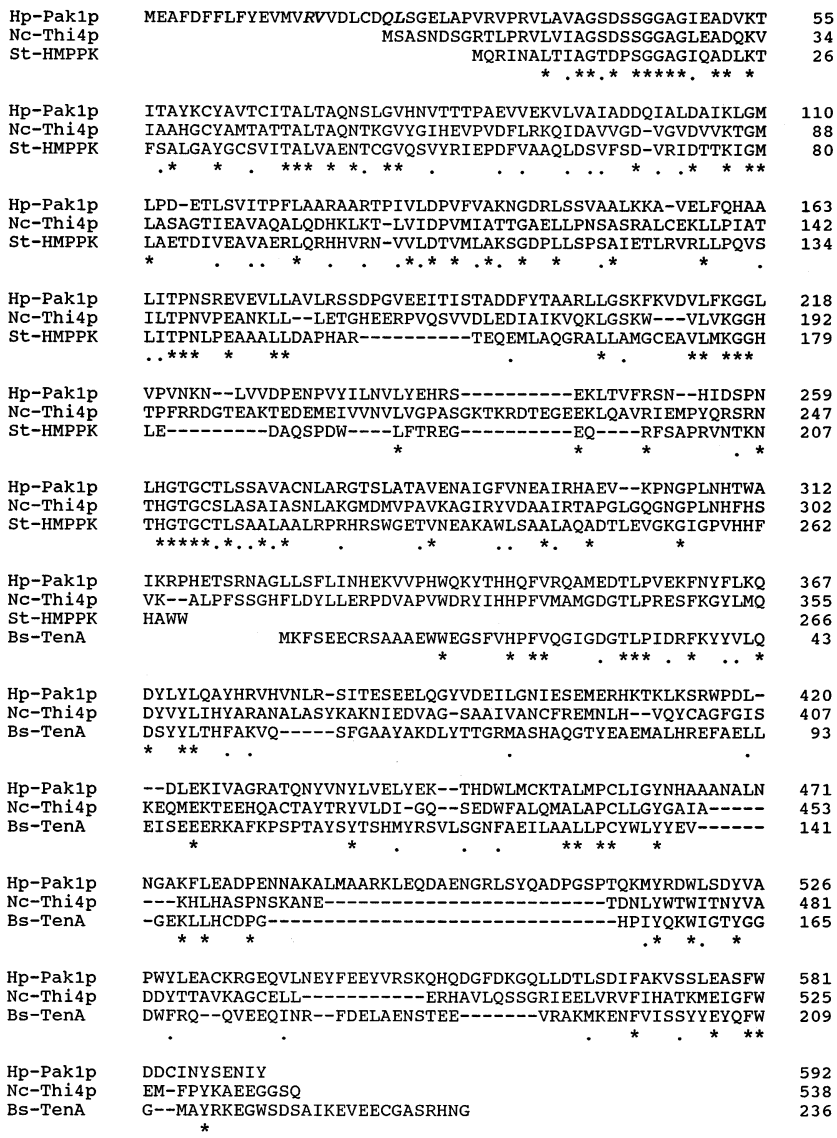

Fig. 3 Comparison of the deduced amino-acid sequences of *H. polymorpha PAK1* with the *N. crassa THI4* protein product (Akiyama and Nakashima 1996), the *S. typhimurium* hydroxymethylpirimidine phosphate-kinase (HMPP-K; Petersen and Downs 1997; SwissProt P55882) and *B. subtilis* tenA (Pang et al. 1991; SwissProt P25052). The *H. polymorpha* and *N. crassa* proteins are homologous over their entire length. HMPP-K is homologous to the N-terminal half of Pak1p, tenA to the C-terminal half of Pak1p. Identical amino acids are indicated by an *asterisk*, similar ones by a *dot*. The putative PTS2 is indicated in italics

tion by the mechanisms detailed before for the turnover of redundant intact peroxisomes in wild-type *H. polymorpha* (Veenhuis et al. 1983; data not shown, compare Fig. 7). Alternatively, AO crystalloids, lacking a surrounding membrane, remained in the cytosol where they often fragmented (data not shown; compare Fig. 7).

Cloning of *per6-210*-complementing DNA

In order to clone the complementing DNA fragment, mutant *per6-210* was transformed with a genomic *H. polymorpha* library. Among approximately 10 000 transformants obtained, four grew well on methanol-containing agar plates (Mut^+-phenotype). These clones appeared to carry overlapping genomic inserts. One clone was selected which contained plasmid DNA with a genomic insert of 5.2 kb. The nucleotide sequence of the complementing portion of this fragment was determined and was deposited at GenBank (accession number AF061946). A BLASTN database search revealed that this fragment corresponds to a region in a large *H. polymorpha* DL1 genomic DNA fragment which was not further characterized, but was cloned by functional complementation of a dihydroxyacetone kinase (DAK)-deficient mutant (Tikhomirova et al. 1988; EMBL accession number X58862).

Analysis of the *per6-210*-complementing sequence revealed two ORFs (Fig. 1). ORF1 encodes a protein of 609 amino acids with a predicted mass of 65 kDa. The protein product was highly similar (34% identity) to the translation product of the *Citrobacter freundii dhaK* gene, encoding the enzyme dihydroxyacetone kinase (DAK; Daniel et al. 1995). In addition two *Saccharomyces cerevisiae* ORFs were found to display strong similarity to ORF1 (51% and 46% identity; SwissProt accession number P43550 and PIR accession number S48327). An alignment of these sequences is given in Fig. 2.

The second ORF encodes a protein of 592 amino acids with a calculated molecular weight of 66.1 kDa (Fig. 3). It showed sequence homology (32% identity) over the entire

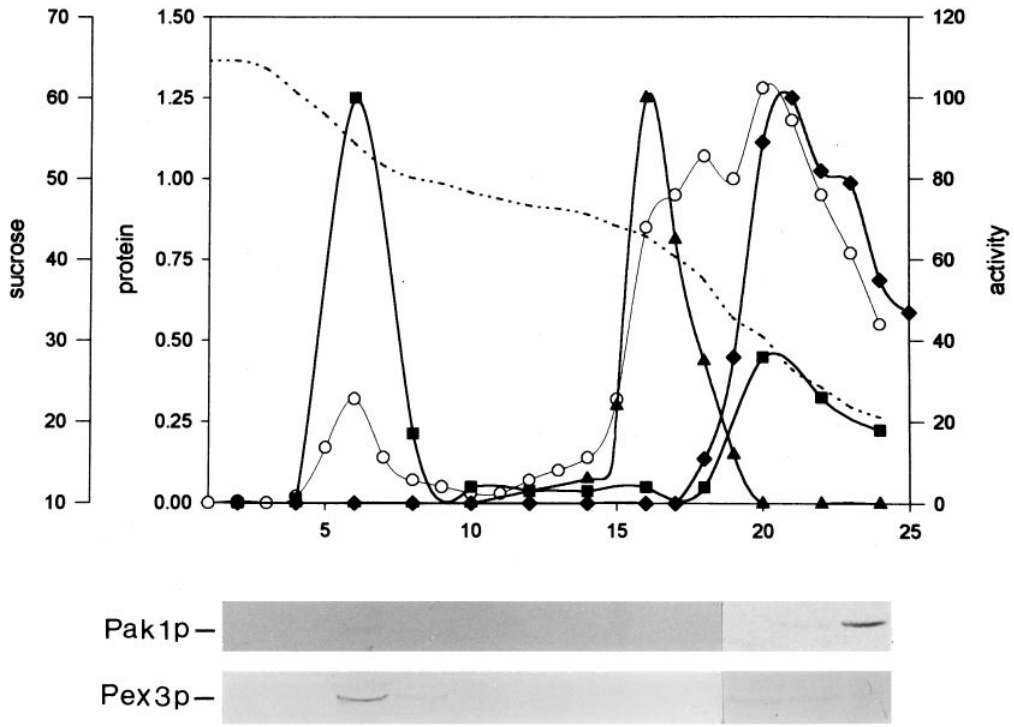

Fig. 4 Sucrose gradient prepared from a post-nuclear supernatant of methanol-grown *H. polymorpha* WT cells. The graph shows the sucrose (– – –) and protein concentration patterns (○—○) and the distribution of the activities of the peroxisomal marker alcohol oxidase (■), mitochondrial cytochrome c oxidase (▲) and dihydroxyacetone kinase (DAK; ◆). The Western blots show the distribution of Pak1p and Pex3p (an integral component of the peroxisomal membrane) in the even fractions 2–24. The bulk of the Pak1p protein is located at the top of the gradient, while a very minor band co-sediments with the peroxisomes (fraction 6, peak fraction of AO activity), where the peroxisomal membrane protein Pex3p is located. Equal portions of the fractions were used for Western blotting. The blots were decorated using α-Pak1p and α-Pex3p antibodies. Sucrose is expressed as a percentage w/w, protein as mg/ml. The enzyme activities are expressed as percentages of the peak activities, which were set at 100

length with the *Neurospora crassa THI4* gene, which is involved in thiamine biosynthesis (Akiyama and Nakashima 1996). Also the putative protein products of three *S. cerevisiae* ORFs [YOL055 C (PIR S66740), YPR121 W (PIR S69014) and YPL258 C (PIR S65289)] are homologous over their entire length (31–37% identity). Their functions are, however, as yet unknown. The N-terminal half of the ORF2 protein product showed similarity to a bacterial protein involved in thiamine biosynthesis (33% identity), namely *Salmonella typhimurium* 4 amino-5-hydroxymethyl-2-methyl pyrimidine phosphate kinase (HMPP-kinase, Swiss Prot P55882; Petersen and Downs 1997). In addition, the C-terminal domain of the ORF2 protein was similar to *Bacillus subtilis* tenA, a putative regulatory component of protein secretion (20% identity; Swiss Prot P25052; Pang et al. 1991).

A hydrophobicity plot of the gene product of ORF2 predicts one possible membrane-spanning region (amino acids 262–292). At the N-terminus a degenerate form of the N-terminal peroxisomal targeting signal PTS2 (RV-X_5-QL) is present. This sequence is identical to the putative PTS2 of *Trypanosoma brucei* glycosomal aldolase (Clayton 1985). We designated ORF2 as *PAK1* and its translation product as Pak1p.

ORF1 encodes the cytosolic enzyme dihydroxyacetone kinase

To test whether ORF1 indeed represented the *H. polymorpha DAK* gene, as suggested by sequence analysis, a strain was constructed in which ORF1 was disrupted (see Fig. 1). Cells of this strain grew normally on mineral media containing glucose, ethanol or glycerol, but were unable to grow on methanol. In addition, cells of this strain were unable to grow on dihydroxyacetone, a compound which requires DAK activity for its metabolism (de Koning et al. 1987). Control cultures of wild-type (WT) or a peroxisome-deficient mutant (Δ*pex8*; Waterham et al. 1994) grew well on DHA. The absence of DAK activity in this strain was confirmed biochemically by enzyme activity measurements which revealed that in crude extracts, prepared from cells of the ORF1 disruption strain grown on glucose or glycerol or incubated for 24 h in methanol-containing media, DAK activity was invariably absent. Based on these findings we conclude that ORF1 encodes the *H. polymorpha* DAK enzyme, and designated it as *DAK*.

The morphology of peroxisomes in cells of the strain in which ORF1 was disrupted (Δ*dak*) was similar to those of normal intact peroxisomes present in WT cells (data not shown). Immunocytochemistry revealed that peroxisomal matrix proteins were, as in WT controls, confined to the organellar matrix (data not shown). Peroxisome disassem-

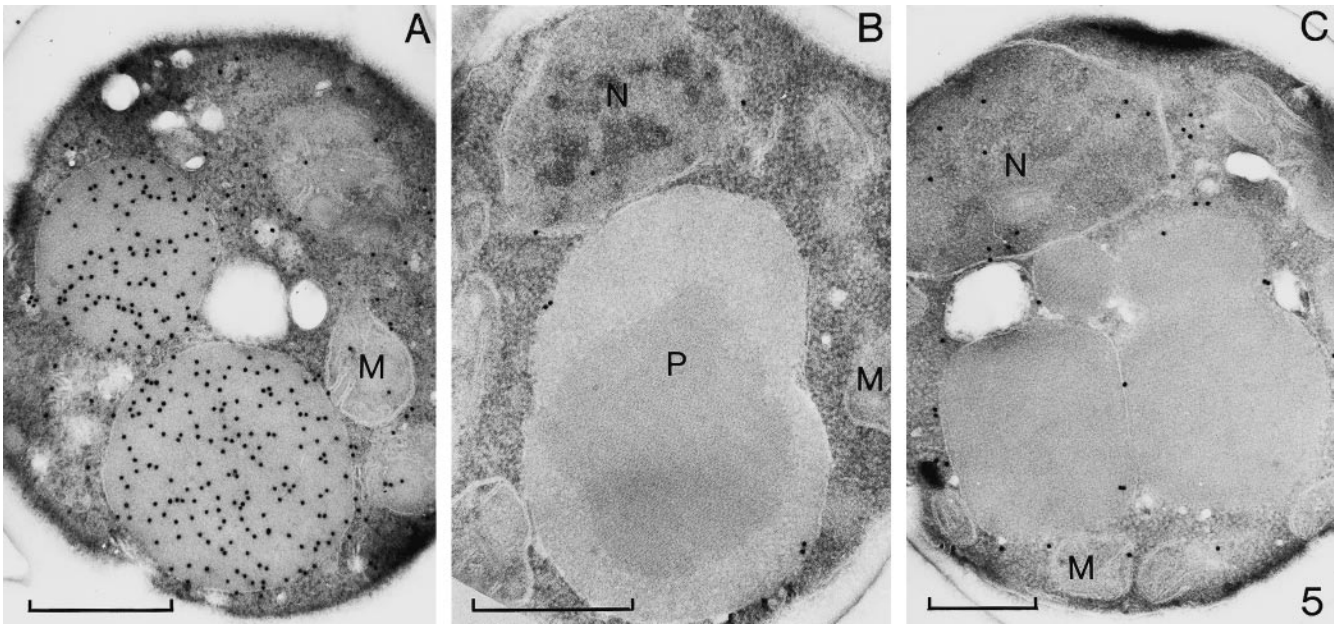

Fig. 5 **A** shows the characteristic labelling pattern after immunocytochemical experiments on methanol-induced Δ*pak1* cells, using α-AO antibodies. The bulk of the labelling is localized on the peroxisomes, while a minor labelling in detectable in the cytosol. Using methanol-grown WT cells (**B**) and α-Pak1p antibodies, labelling is localized on the peroxisomal membrane. In identical experiments on WT::P_{AOX}.*PAK1* cells (**C**) peroxisomal labelling is increased, while labelling is also observed in the cytosol. Aldehyde fixation. Abbreviations: *M* mitochondrion, *N* nucleus, *P* peroxisome. The marker represents 0.5 μm

bly or increased organellar turnover, typical for *per6-210* cells, was never observed in Δ*dak* cells.

The subcellular location of DAK was determined by cell-fractionation experiments (Fig. 4). Sucrose-density centrifugation of a post-nuclear supernatant obtained from homogenates of methanol-grown WT *H. polymorpha* resulted in a clear separation of peroxisomes (marker protein AO, fraction 6), mitochondria (marker protein cytochrome c oxidase, fraction 16) and soluble proteins (fractions 23–25). As shown in Fig. 4, DAK activity was confined to the upper part of the gradient, which indicates that it is a cytosolic protein.

Characterization of a *PAK1* disruption mutant

In order to test whether the peroxisomal aberrations observed in *per6-210* cells were related to a deficiency in the protein product of the second ORF (*PAK1*), a *PAK1* disruption mutant was constructed (Fig. 1). Physiological studies revealed that the resulting strain (Δ*pak1*) grew normally on methanol and peroxisomes were formed in methanol-grown Δ*pak1* cells. Occasionally, peroxisomal abnormalities like those observed in *per6-210* cells were encountered, especially in cells from batch cultures in the late exponential and stationary growth phase. Under these conditions immunocytochemical experiments revealed that the peroxisomal matrix proteins were predominantly peroxisomal but were also present in the cytosol (Fig. 5A, AO; DHAS not shown). In WT control cells labelling was invariably confined to the peroxisomal matrix when the same antisera were used (data not shown; see Douma et al. 1985).

Pak1p is a low abundant, methanol-inducible protein

In order to obtain insight into the function of the *PAK1* gene product, the levels of Pak1p were determined in cells grown under various conditions. For this purpose, Pak1p antibodies were raised using a Pak1p-MBP fusion protein synthesized in *E. coli*. With these antibodies, a single protein band of approximately 68 kDa was observed in Western blots prepared from crude extracts of methanol-grown *H. polymorpha* WT cells (Fig. 6A). The apparent molecular weight of the protein was in good agreement with the calculated mass of the protein (66.1 kDa). The intensity of the band was very low and only detectable using chemiluminescence techniques. In extracts prepared from Δ*pak1* cells the 68 kDa-band was invariably absent. To increase the Pak1p protein level, a strain was constructed which contained an additional copy of *PAK1* under the control of the strong AO promoter. In Western blots of crude extracts prepared from methanol-induced cells of this strain, the intensity of the 68-kDa band had significantly increased. We concluded from these data that the 68-kDa band represented Pak1p and that the antiserum specifically recognized Pak1p, but no other *H. polymorpha* proteins.

The induction of Pak1p was subsequently analyzed in crude extracts of variously grown *H. polymorpha* WT cells by Western blotting using α-Pak1p antibodies. The results, presented in Fig. 6B, indicate that synthesis of Pak1p is enhanced in methanol-grown cells compared to glucose-grown cells. In cells grown on glucose in the presence of thiamine, Pak1p was not detected, but a faint band became

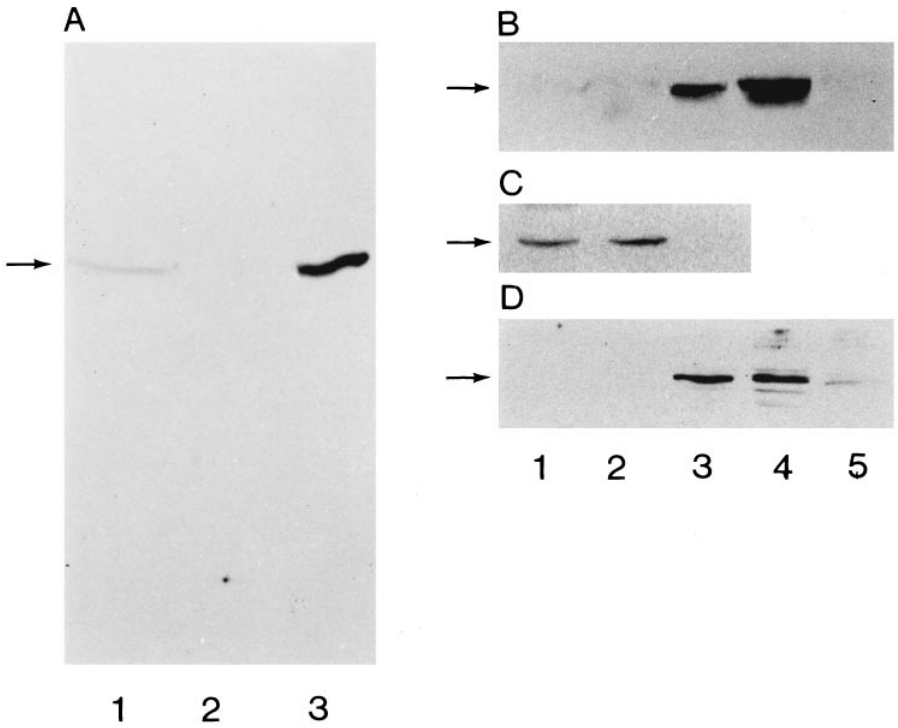

Fig. 6A–D Protein levels and localization of Pak1p. **A** shows the specificity of the polyclonal α-Pak1p antiserum. Crude extracts prepared from methanol-induced WT cells (*lane 1*; 30 µg protein), Δ*pak1* cells (*lane 2*; 30 µg protein) or a strain over-producing Pak1p (*lane 3*; 7 µg protein) were used for SDS-PAGE followed by Western blotting. **B** Western blots prepared of crude extracts of WT *H. polymorpha* grown under various conditions showing the protein levels of Pak1p. *Lane 1* WT glucose; *lane 2* WT glucose, thiamine-limitation; *lane 3* WT methanol; *lane 4* WT methanol, thiamine-limitation; *lane 5* Δ*pak1*; 40 µg of protein was loaded on each lane. **C** A 30 000-g organellar pellet, prepared from homogenates of methanol-grown WT cells (*lane 1*), was subjected to high-salt extraction by incubation in 0.5 M NaCl for 30 min at 0°C. The soluble proteins (*lane 2*) and membrane-bound proteins (*lane 3*) were separated by centrifugation at 100 000 g. Equal portions of the pellet and soluble fractions were loaded per lane. **D** Pak1p levels in crude extracts of different *H. polymorpha* strains. *Lane 1* Δ*pak1*, *lane 2* Δ*dak* Δ*pak1*, *lane 3* Δ*dak*, *lane 4* WT and *lane 5 per6-210;* 30 µg protein was loaded on each lane. All blots were decorated using anti-Pak1p antiserum; protein bands were detected using chemiluminescence

visible when crude extracts were used prepared from cells grown on glucose under thiamine limitation. An increase in Pak1p levels due to thiamine limitation was more evident when cells grown on methanol under conditions of thiamine excess were compared with cells grown on methanol under thiamine-limitation conditions (Fig. 6B).

Subcellular location of Pak1p

Immunocytochemical experiments on ultrathin sections of WT *H. polymorpha* cells, using α-Pak1p antibodies, resulted in a specific labelling of the peroxisomal membrane. However, the labelling intensities were very low (generally 2–3 gold particles/organelle; Fig. 5B), in agreement with the very low levels of Pak1p in WT cells. The ultrastructural data, which suggested that Pak1p is associated with the peroxisomal membrane, could not be confirmed biochemically using cell-fractionation experiments. In sucrose gradients, prepared from a post-nuclear supernatant of methanol-grown WT cells, a very minor portion of Pak1p co-sedimented with the peroxisomes (fraction 6) while the bulk of the protein was found on top of the gradient (Fig. 4). Upon extraction of an enriched peroxisomal fraction (30 000 g pellet) by high salt, all sedimentable Pak1p became solubilized. This suggests that the organellar Pak1p is loosely associated with the outer surface of the organellar membrane (Fig. 6C). After over-production of Pak1p, α-Pak1p-specific labelling was predominantly localized at the peroxisomal membrane, but was also present in the cytosol (Fig. 5C). The latter results should however be interpreted with care, because over-production of Pak1p may alter its subcellular location. Taken together these data suggest that Pak1p may have a dual location and is present both at the peroxisomal membrane and in the cytosol.

Per6-210 is affected in both *DAK* and Pak1p

The phenotypes of the single Δ*dak* and Δ*pak1* mutants differed from that of the original *per6-210* mutant. Analysis of the original mutant *per6-210* revealed that this strain is unable to grow on DHA and lacked DAK activity, indicating that the *DAK* gene is inactivated. In addition, methanol-induced *per6-210* cells contain strongly reduced Pak1p levels (Fig. 6D), which suggests that expression of *PAK1* may be disturbed as well. Additional evidence that *per6-210* is defective in both gene products came from

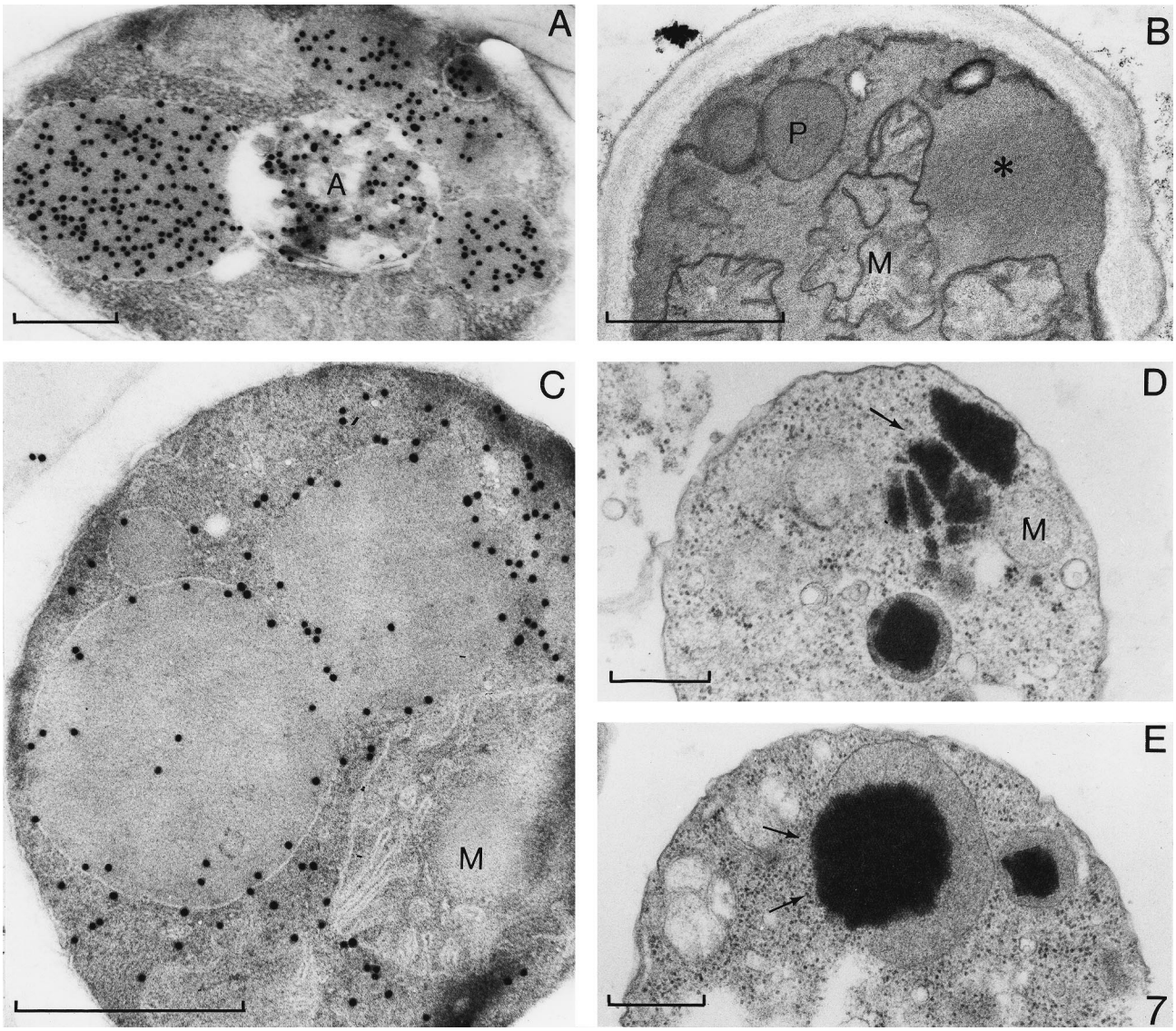

Fig. 7A–E Methanol-induced cells of the Δ*dak* Δ*pak1* double deletion strain. **A** shows an autophagic vacuole, containing peroxisomal AO (α-AO), **B** shows a typical example of the peroxisome proliferation and the presence of an AO crystalloid (*) in these cells. We assume that these crystalloids are due to a generalized import defect, as shown for catalase protein (**C**; anti-CAT). AO crystalloids, resulting from ruptured peroxisomes (**E**; *arrows*) often disintegrated (**D**; *arrow*). (**A** and **C**: aldehyde, Unicryl, uranyl acetate; **B**: $KMnO_4$; **D–E**: glutaraldehyde/OsO_4). Abbreviations: *A* autophagic vacuole, *M* mitochondrion, *P* peroxisome

studies on a constructed double mutant in which major parts of both the *DAK* and *PAK1* genes were deleted (Δ*dak* Δ*pak1*; Fig. 1). As expected, Δ*dak* Δ*pak1* cells lacked DAK activity and were unable to grow on methanol or DHA. Also Pak1p was absent in these cells (Fig. 6D). Ultrastructural analysis revealed that the peroxisomal morphology in methanol-induced cells of the double mutant was similar to that of the original *per6-210* mutant (Fig. 7).

A single mutation in *per6-210* may affect both *DAK* activity and *PAK1* expression

The smallest DNA fragment still able to functionally complement the *per6-210* mutation is a 3.2-kb *PvuII* fragment (nt 1357–4422) containing the 3′ half of ORF1 (encoding the C-terminal 310 amino acids of DAK) and the complete ORF2 (see Fig. 1). Most likely, functional complementation was due to integration/recombination events. Sequence analysis of a cloned 1.1-kb fragment which included the 3′ half of *DAK* and the 5′ end of *PAK1*, revealed that in *per6-210* a G-to-A transition had occurred in nucleotide 1658. This mutation resulted in the substitution of the conserved glycine 401 of DAK into glutamic acid (Fig. 2). This mutation is present only 670 bp upstream of the start codon of *PAK1* and hence could affect the regulatory elements of the *PAK1* promoter.

Taken together, these data support the view that possibly both genes may be inactivated in *per6-210* due to a single mutation.

Physiological studies

To gain further insight in the role of DAK and Pak1p in methanol metabolism, physiological studies were carried out using glucose-limited chemostat cultures of Δ*pak1*, Δ*dak* or Δ*dak* Δ*pak1* cells. In WT *H. polymorpha* the addition of methanol as a second carbon source to glucose-limited chemostat cultures results in an increase in yield due to the simultaneous utilization of glucose and methanol under these conditions (van der Klei et al. 1991). In experiments, in which 0.2% methanol was added to a glucose-limited chemostat culture of the Δ*dak* Δ*pak1* double mutant the yield of the culture increased only slightly (0.4 D_{660} units). In similar experiments, carried out with the single mutant Δ*pak1*, the increase in biomass was considerably higher (1.9 D_{660} units) and comparable to the values observed in cultures of WT cells (van der Klei et al. 1991). In case of the Δ*dak* mutant an intermediate increase was observed (0.8 D_{660} units). Hence, the low increase observed in Δ*dak* Δ*pak1* cultures most likely reflects the combined defects of both DAK and Pak1p. In vivo ^{31}P NMR studies on methanol-induced cells demonstrated that the reduction in yields of Δ*dak* Δ*pak1* cultures were not due to specific energetic disadvantages caused by damaged or non-functional peroxisomes, as have been observed in various *pex* strains (van der Klei et al. 1991). In both cultures normal ATP levels were observed and the inorganic phosphate peaks, indicating the acidic nature of the peroxisomal lumen (pH approximately 5.8–6.0), were normally found (data not shown, see Nicolay et al. 1987). These results therefore confirm the morphological data which indicated that in Δ*dak* Δ*pak1* cells at least a substantial portion of the peroxisomal population is intact and physiologically active.

An alternative explanation could be that, due to the absence of DAK activity, the cells start to produce dihydroxyacetone (DHA). Indeed, after the addition of methanol up to 12 mM, DHA was detectable in the growth medium. Hence, part of the formaldehyde produced from methanol oxidation is assimilated by DHAS, resulting in the formation and subsequent secretion of DHA and, as such, can account for the drop in yield compared to WT controls.

Discussion

In this paper we describe the *H. polymorpha per6-210* mutant, which is impaired in respect of growth on methanol (Mut$^-$) and characterized by the presence of aberrant peroxisomes. The complementing DNA fragment contained two complete ORFs. ORF1 encodes the *H. polymorpha* enzyme dihydroxyacetone kinase (DAK), while ORF2 codes for a protein, designated Pak1p, which plays a role in peroxisome integrity, especially in the later stages of growth of cells on methanol. So far, only a single gene encoding DAK has previously been characterized, namely *dhaK* of the prokaryote *Citrobacter freundii* (Daniel et al. 1995), which shows 34% identity to the *H. polymorpha* protein. The *H. polymorpha* DAK protein has been purified to homogeneity and characterized by Kato et al. (1988), who demonstrated that the enzyme was a homodimer composed of two identical subunits of 72 kDa, which is in fair agreement with the calculated molecular weight of the *H. polymorpha DAK* protein product (65 kDa). In *H. polymorpha*, DAK is a cytosolic enzyme of the xylulose-5-phosphate (Xu5P) pathway, essential for the assimilation of formaldehyde generated from methanol. The first step in the assimilation pathway is the conversion of formaldehyde and Xu5P into dihydroxyacetone (DHA) and glyceraldehyde phosphate (GAP), mediated by peroxisomal dihydroxyacetone synthase (DHAS; Douma et al. 1985). Subsequently, DHA is phosphorylated in the cytosol by DAK, resulting in the formation of dihydroxyacetone phosphate (DHAP). Then, DHAP and GAP are converted into fructose-bis-phosphate (FBP) and subsequently, in a series of rearrangement reactions, into GAP, used for biosynthetic processes, and Xu5P, to replenish the Xu5P essential for the initial – peroxisomal – condensation reaction (van der Klei et al. 1991). Prevention of DHA phosphorylation in a Δ*dak* strain inhibits operation of the Xu5P pathway (and thus growth on methanol as a sole carbon and energy source) and explains the Mut$^-$ phenotype and the observed secretion of DHA in methanol cultures of these strains.

As expected, in chemostat cultures of Δ*dak* cells on glucose/methanol mixtures, methanol was fully used because AO was normally active. However, the addition of methanol as second substrate resulted in a significantly lower increase in biomass, as observed in WT controls (Δ*dak* 0.8 and WT 2.0 D_{660} units, respectively). This can be explained by the fact that glucose-derived moieties have to replenish the DHAP, which cannot be formed by the normal Xu5P pathway in Δ*dak* cells.

The effects of the *PAK1* disruption on the efficiency of methanol metabolism and peroxisome integrity was particularly prominent in a Δ*dak* Δ*pak1* double mutant during the growth of cells in a chemostat on glucose/methanol mixtures. Essentially, the phenotype of methanol-induced *per6-210* cells (Mut$^-$, peroxisomal defects, no DAK activity) was akin to the constructed Δ*dak* Δ*pak1* cells and thus most likely represents the combined effects of the disruption of either of the *DAK* or the *PAK1* genes alone. This view is supported by the observed reduced Pak1p levels in *per6-210* cells which might be explained by a point mutation in the coding region of *DAK*, which supposedly overlaps with the promoter region of *PAK1*.

In contrast to DAK, the function of Pak1p is less clear. The sequence analyses revealed that the N-terminal half of Pak1p and the protein product of *N. crassa THI4*, essential for thiamine biosynthesis in this organism, are homologous to *Salmonella typhimurium* hydroxymethyl pyrimidine kinase, also involved in thiamine biosynthesis. Thiamine is the precursor of thiamine pyrophosphate (TPP), which is the co-factor of α-keto acid decarboxylase, α-keto acid oxidase and several transketolases (Begley 1996), including *H. polymorpha* DHAS (Bystrykh et al. 1990), which may explain the increased level of Pak1p in methanol-grown *H. polymorpha* cells compared to glucose-

grown cells. Also, *PAK1* expression is partially repressed by thiamine, a phenomenon which has been reported for several, but not all, genes involved in thiamine biosynthesis (Begley 1996).

Although the above findings suggest a role of Pak1p in thiamine biosynthesis, this function is not immediately clear in the case of *H. polymorpha* because this organism is not able to synthesize thiamine, but is dependent on the addition of this vitamin to the cultivation medium for growth. A clue to the function of Pak1p may be deduced from studies on thiamine biosynthesis in *S. cerevisiae* and *S. pombe*. This view is based on the fact that the subcellular locations of the known yeast enzymes involved in thiamine biosynthesis are not yet known. However, TPP-dependent enzymes are present in various cell compartments (e.g. mitochondria, peroxisomes). This therefore implies that TPP (or precursor forms) are either transported across intracellular membranes or else synthesized in different subcellular compartments. Interestingly, in *S. cerevisiae* three Pak1p homologues have been found which may be located at different sites in the cell. If so, it can be envisaged that, due to transport barriers, defects in the synthesis at one site can not be (fully) restored by thiamine added to the growth medium or synthesized in another organelle.

This may serve as an explanation as to why in *H. polymorpha* a defect in a gene required for thiamine biosynthesis still shows a phenotype even when thiamine is supplemented in the growth medium. Decreased TPP levels in *PAK1* disruption strains may explain the mislocation of a portion of the peroxisomal enzymes in the cytosol. The assumption that limitation of a co-factor of a peroxisomal enzyme may affect its subcellular location is not without precedent. Studies on a riboflavin (Rf)-auxotrophic mutant of *H. polymorpha* showed that the limitation of Rf (and thus FAD, the AO co-factor) resulted in the mislocation of a portion of the AO protein and other peroxisomal matrix enzymes in the cytosol (Evers et al. 1994, 1996). A similar phenomenon may explain the observed cytosolic portion of peroxisomal enzymes in a Pak1p-deficient environment, namely an imbalance in the amount of newly synthesized DHAS protein and its co-factor TPP.

Taken these data together, we propose that Pak1p plays a role in thiamine biosynthesis and is possibly involved in the generation of adequate amounts of the co-factor TPP for the peroxisomal enzyme DHAS.

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